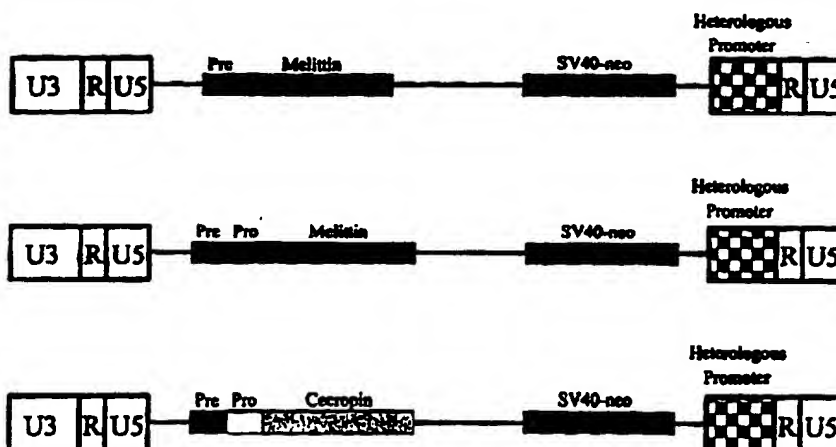




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(54) Title: VECTORS CARRYING THERAPEUTIC GENES ENCODING ANTIMICROBIAL PEPTIDES FOR GENE THERAPY

The Melittin & Cecropin Retroviral Vector Constructs

(57) Abstract

The present invention relates to retroviral vectors carrying sequences encoding naturally occurring, antimicrobial peptides or derivatives thereof for the treatment of mammalian tumours, viral infections such as HIV infection and bacterial and fungal infections. In particular the present invention relates to retroviral vectors which undergo promoter conversion (Procon vectors) carrying such sequences. Since these vectors also carry tumour or virus specific regulatory elements, the therapeutic antimicrobial peptide will be delivered and expressed only in relevant, affected cells and not in innocent bystander cells.

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VECTORS CARRYING THERAPEUTIC GENES ENCODING ANTIMICROBIAL PEPTIDES FOR GENE THERAPY

The present invention relates to recombinant vectors carrying sequences encoding naturally occurring antimicrobial peptides or derivatives thereof for the treatment of mammalian tumours and viral infections such as HIV infections and bacterial and fungal infections. In particular the present invention relates to retroviral vectors. Furthermore, the present invention relates to retroviral vectors which undergo promoter conversion (Procon vectors) carrying such sequences. Since these vectors also carry tumour or virus specific regulatory elements, the therapeutic antimicrobial peptide will be delivered and expressed only in relevant, affected cells and not in innocent bystander cells.

Background of the Invention

The introduction of therapeutic genes into cells for the treatment of diseases as diverse as those resulting from genetic defects, cancer and viral infections is the major aim of gene therapy. Cancer and diseases such as AIDS resulting from infection with human immunodeficiency virus (HIV) are particularly difficult to treat even though a number of clinical protocols are presently underway that use gene therapeutical approaches. The amphipathic peptide melittin, the major component of bee venom, has been shown to have selective anti-cancer (Sharma, 1992; Sharma, 1993) and anti-HIV activity (Wachinger et al., 1992); U.S. Patent No. 4,822,608 of Benton et al. and WO patent application 91/08753 of Erle et al. both relate to these therapeutic properties.

U.S. Patent No. 4,822,608 issued to Benton et al. on April 18, 1989 and entitled "METHODS AND COMPOSITIONS FOR THE TREATMENT OF MAMMALIAN INFECTIONS EMPLOYING MEDICAMENTS COMPRISING HYMENOPTERA VENOM OR PROTEINACEOUS OR POLYPEPTIDE COMPONENTS THEREOF" teaches that secondary agents derived from nature such as hymenoptera venom or proteinaceous or polypeptide components thereof has a potentiating effect on antibacterial agents. This reference further suggests that such compositions may

also have increased anti-viral, carcinostatic and anti-carcinogenic effects on various maladies. More particularly, the reference to Benton et al. discloses the use of melittin which is the main component of honey bee toxin, in combination with assorted antibiotic agent as having antibacterial activity against predetermined infections. Further this reference teaches that a synergistic benefit may be achieved by the combination of the melittin and assorted antibiotics in various therapeutically effective amounts.

WO patent application 91/08753 of Erfle et al. relates to a method and composition for the treatment of mammalian HIV infections, and more particularly to such a method and composition for treating mammalian HIV infections which employs hymenoptera venom, or proteinaceous or polypeptide components thereof and which is introduced into the mammalian hosts and which are individually operable to restrict or substantially inhibit the virus replication in the HIV infected cells of the mammal.

In these studies purified melittin peptide was given to cells in culture which, though useful for experimental purposes, is not relevant for therapy. Even in vivo administration of purified melittin protein (for example i.v.) is probably not advisable because of the relatively high concentrations and repeated doses that would be required to maintain therapeutic levels. Further, since this kind of generalized delivery would result in the amphipathic peptide reaching not only target cells but also other cells, thereby potentially resulting in nondesirable side effects, it would be advantageous to be able to target the delivery of melittin or other antimicrobial peptides, in particular the melittin peptide and the peptides mentioned below.

A second class of therapeutic genes of interest are the cecropins isolated from the pupae of giant silk moths (Boman, 1995, Hultmark et al., 1980). There are three principal cecropins, A, B and D with a similar structure to melittin (Hultmark et al., 1982). Cecropins A and B show specific antibacterial activity without any apparent ill effects for mammalian cells (Steiner et al., 1981). Recently Moore and coworkers have shown that the cecropin B, P and Shiva-1 antibacterial peptides show anticancer activity against a variety of tumour cell lines (Moore et al. 1994).

Cecropins were first isolated from the hemolymph of the giant silk moth, *Hyalophora cecropia*, following induction by live non-pathogenic bacteria. The principal insect cecropins (A, B and D) are 35 to 37 residues long, devoid of cysteine and have a strongly basic N-terminus linked to a neutral C-terminus by a flexible glycine-proline link. The overall structure deduced by NMR for cecropin A is two nearly perfect amphipathic segments joined by a Gly-Pro hinge. A cecropin-like 31-residue peptide (cecropin P₁), isolated from the small intestine of a pig (Lee et al., 1989), suggests that the cecropins may be widespread throughout the animal kingdom. The mechanism of action of the cecropins is thought to involve channel formation in membranes and subsequent lysis.

SB-37 (a close cecropin B analogue) and Shiva-1 (a cecropin B analogue that shares about 40% sequence homology and maintains the same charge distribution and hydrophobicity as the peptide) have been shown to lyse several mammalian leukemia and lymphoma cell lines in vitro. The publication of Moore et al., 1994 is incorporated herein by reference for complete disclosure. Similar antitumour effects have been demonstrated for the magainins, a related group of antimicrobial peptides (Cruciani et al., 1991; Ohaski et al., 1992).

The use of retroviral vectors (RV) for gene therapy has received much attention and currently is the method of choice for the transferral of therapeutic genes in a variety of approved protocols both in the USA and in Europe (Kotani *et al.*, 1994). However most of these protocols require that the infection of target cells with the RV carrying the therapeutic gene occurs *in vitro*, and successfully infected cells are then returned to the affected individual (Rosenberg *et al.*, 1992; for a review see Anderson, 1992). Such *ex vivo* gene therapy protocols are ideal for correction of medical conditions in which the target cell population can be easily isolated (e.g. lymphocytes). Additionally the *ex vivo* infection of target cells allows the administration of large quantities of concentrated virus which can be rigorously safety tested before use.

Unfortunately, only a fraction of the possible applications for gene therapy involve target cells that can be easily isolated, cultured and then reintroduced. Additionally,

the complex technology and associated high costs of *ex vivo* gene therapy effectively preclude its disseminated use world-wide. Future facile and cost-effective gene therapy will require an *in vivo* approach in which the viral vector, or cells producing the viral vector, are directly administered to the patient in the form of an injection or simple implantation of RV producing cells.

This kind of *in vivo* approach, of course, introduces a variety of new problems. First of all, and above all, safety considerations have to be addressed. Virus will be produced, possibly from an implantation of virus producing cells, and there will be no opportunity to precheck the produced virus. It is important to be aware of the finite risk involved in the use of such systems, as well as trying to produce new systems that minimize this risk.

The essentially random integration of the proviral form of the retroviral genome into the genome of the infected cell led to the identification of a number of cellular proto-oncogenes by virtue of their insertional activation (Varmus, 1988). The possibility that a similar mechanism may cause cancers in patients treated with RVs carrying therapeutic genes intended to treat other pre-existent medical conditions, has posed a recurring ethical problem. Most researchers would agree that the probability of a replication defective RV, such as all those currently used, integrating into or near a cellular gene involving in controlling cell proliferation is vanishingly small. However it is generally also assumed that the explosive expansion of a population of replication competent retrovirus from a single infection event, will eventually provide enough integration events to make such a phenotypic integration a very real possibility.

Retroviral vector systems are optimized to minimize the chance of replication competent virus being present. However it has been well documented that recombination events between components of the RV system can lead to the generation of potentially pathogenic replication competent virus and a number of generations of vector systems have been constructed to minimize this risk of recombination (reviewed in Salmons and Günzburg, 1993). However little is known about the finite probability of these events. Since it will never be possible to reduce

the risk associated with this or other viral vector systems to zero, an informed risk-benefit decision will always have to be taken. Thus it becomes very important to empirically determine the chance of (1) insertional disruption or activation of single genes by retrovirus integration and (2) the risk of generation of replication competent virus by recombination in current generations of packaging cell lines. A detailed examination of the mechanism by which these events occur will also allow the construction of new types of system designed to limit these events.

A further consideration for practical *in vivo* gene therapy, both from safety considerations as well as from an efficiency and from a purely practical point of view, is the targeting of RVs. It is clear that therapeutic genes carried by vectors should not be indiscriminately expressed in all tissues and cells, but rather only in the requisite target cell. This is especially important if the genes to be transferred are toxin genes aimed at ablating specific tumour cells. Ablation of other, nontarget cells would obviously be very undesirable. Targeting of the expression of carried therapeutic genes can be achieved by a variety of means.

Retroviral vector systems consist of two components (Fig. 1):

1) the retroviral vector itself is a modified retrovirus (vector plasmid) in which the genes encoding for the viral proteins have been replaced by therapeutic genes optionally including marker genes to be transferred to the target cell. Since the replacement of the genes encoding for the viral proteins effectively cripples the virus it must be rescued by the second component in the system which provides the missing viral proteins to the modified retrovirus.

The second component is:

2) a cell line that produces large quantities of the viral proteins, however lacks the ability to produce replication competent virus. This cell line is known as the packaging cell line and consists of a cell line transfected with a second plasmid carrying the genes enabling the modified retroviral vector to be packaged. This

plasmid directs the synthesis of the necessary viral proteins required for virion production.

To generate the packaged vector, the vector plasmid is transfected into the packaging cell line. Under these conditions the modified retroviral genome including the inserted therapeutic and optional marker genes is transcribed from the vector plasmid and packaged into the modified retroviral particles (recombinant viral particles). A cell infected with such a recombinant viral particle cannot produce new vector virus since no viral proteins are present in these cells. However the vector carrying the therapeutic and marker genes is present and these can now be expressed in the infected cell.

Objects of the Invention

It is an object of the present invention to provide a novel therapeutic agent with antitumour, antiviral, antibacterial and/or antifungal activities.

It is a further object of the present invention to provide a novel therapeutic agent with high selectivity for selected target cells and reduced undesirable side effects.

The Invention

To achieve the foregoing and other objects, the present invention provides a recombinant vector for introducing into an eucaryotic cell DNA, the vector comprising, in operable linkage, a) the DNA of or corresponding to at least a portion of a vector, which portion is capable of infecting and directing the expression in the target cells; and b) one or more coding sequences wherein at least one sequence encodes for at least one naturally occurring therapeutic antimicrobial peptide or a derivative thereof for the treatment of at least one disease, selected from mammalian tumours, viral, bacterial and fungal infections.

Said sequence encoding a naturally occurring therapeutic antimicrobial peptide or derivative thereof encodes for the amino acid sequences of all, part, an analogue, homologue, recombinant or combination thereof of such antimicrobial peptide.

Said sequences comprise preferably also non-coding sequences.

The antimicrobial peptides or derivatives thereof include but are not limited to those encoding melittin, the various cecropins and magainins. Further included are the apidaecin and defensin peptides or derivatives thereof. These genes may be expressed in their preproform or alternatively in a genetically engineered preform or in another form which renders a biological active peptide or a derivative thereof.

Said sequence is preferably a recombinant molecule coding for the amino acid sequences of all, part, and analogue, homologue, derivative, recombinant or combination thereof of the melittin, cecropin, magainin, apidaecin and defensin genes.

As discussed in detail in the prior art reference to Benton et al., melittin, the main component in honey bee toxin is a polypeptide which includes substantially 26 amino acid residues. These amino acid residues include, Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln. Moreover, melittin analogues wherein at least the last six (C-terminal) amino acids is altered and replaced by six glycine residues appear to have a therapeutic benefit similar to melittin, these amino acid analogues having a structure of Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Gly-Gly-Gly-Gly-Gly-Gly. The use of melittin for treatment of HIV infections is disclosed in WO patent application 91/08753 of Erle et al., which is incorporated herein by reference for complete disclosure.

According to a further preferred embodiment of the invention, the sequence encoding a therapeutic antimicrobial peptide is coding for a hymenoptera venom, at least one active protein component of a hymenoptera venom, at least one polypeptide component of a hymenoptera venom, and mixtures thereof.

The hymenoptera gene is preferably selected from the group consisting of genes coding for honeybee venom, bumble bee venom, yellow jacket venom, bald-faced hornet venom, active protein components of said venom, active protein components of said venom, and mixtures thereof.

Furthermore, the structural analogues of melittin include an amphiphilic helix with or without signal peptide and activation domains.

In a preferred embodiment, the recombinant vector is selected from viral and plasmid vectors. Examples for viral vectors are RNA and DNA virus vectors. A particularly preferred RNA virus vector is a retrovirus vector, more particularly a procon vector. Examples for DNA virus vectors are adenoviruses, adenovirus associated viruses and herpes viruses derived vectors. The plasmid vectors include all eucaryotic expression vectors.

In a preferred embodiment of the invention, the recombinant vector is a retroviral vector.

The retroviral genome consists of an RNA molecule with the structure R-U5-gag-pol-env-U3-R (Fig. 1). During the process of reverse transcription, the U5 region is duplicated and placed at the right hand end of the generated DNA molecule, whilst the U3 region is duplicated and placed at the left hand end of the generated DNA molecule (Fig. 1). The resulting structure U3-R-U5 is called LTR (Long Terminal Repeat) and is thus identical and repeated at both ends of the DNA structure or provirus. The U3 region at the left hand end of the provirus harbours the promoter. This promoter drives the synthesis of an RNA transcript initiating at the boundary between the left hand U3 and R regions and terminating at the boundary between the right hand R and U5 region (Fig. 1). This RNA is packaged into retroviral particles and transported into the target cell to be infected. In the target cell the RNA genome is again reverse transcribed as described above.

According to another embodiment of the invention a promoter conversion vector (procon vector) can be constructed in which the righthand U3 region is altered (Fig. 3), but the normal lefthand U3 structure is maintained (Fig. 3); the vector can be normally transcribed into RNA utilizing the normal retroviral promoter located within the left hand U3 region (Fig. 3). However the generated RNA will only contain the altered righthand U3 structure. In the infected target cell, after reverse transcription, this altered U3 structure will be placed at both ends of the retroviral structure (Fig. 3).

If the altered region carries a polylinker instead of the U3 region then any promoter, including those directing tissue specific expression such as the WAP promoter can be easily inserted. This promoter will then be utilized exclusively in the target cell for expression of linked genes carried by the retroviral vector. Alternatively or additionally DNA segments homologous to one or more cellular sequences can be inserted into the polylinker for the purpose of gene targeting.

In the packaging cell line the expression of the retroviral vector is thus regulated by the normal unselective retroviral promoter (Fig. 3). However as soon as the vector enters the target cell promoter conversion occurs, and the therapeutic genes are expressed from a tissue specific promoter of choice introduced into the polylinker (Fig. 3). Not only can virtually any tissue specific promoter be included in the system, providing for the selective targeting of a wide variety of different cell types, but additionally, following the conversion event, the structure and properties of the retroviral vector no longer resembles that of a virus. This, of course, has extremely important consequences from a safety point of view, since ordinary or state of the art retroviral vectors readily undergo genetic recombination with the packaging vector to produce potentially pathogenic viruses. Promoter conversion (Procon) vectors do not resemble retroviruses because they no longer carry U3 retroviral promoters after conversion thus reducing the possibility of genetic recombination. inserted into the polylinker for the purposes of gene targeting.

For a complete disclosure of the procon vectors, the content of the Danish application DK 1017/94, filed on September 2, 1994 is completely included within the present application.

Thus in a further preferred embodiment of the invention a retroviral vector undergoing promoter conversion (procon vector) is provided comprising a 5' LTR region of the structure U3-R-U5; one or more coding sequences wherein at least one of said coding sequences encodes a naturally occurring antimicrobial peptide or a derivative thereof (part, an analogue, homologue, recombinants or a combination thereof of such antimicrobial gene) for the treatment of at least one disease selected from mammalian tumours, viral infections, bacterial infections and fungal infections; and a 3'LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence, followed by the R and U5 region.

And in a further preferred embodiment a retroviral vector is provided wherein said retrovirus vector includes, in operable linkage, a 5' LTR region and a 3' LTR region, said 5' LTR region comprising the structure U3-R-U5 and said 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by one or more of said coding sequences wherein at least one sequence encodes for at least one naturally occurring therapeutic antimicrobial peptide or a derivative thereof for the treatment of at least one disease, selected from mammalian tumours, viral, bacterial and fungal infections expressed from either the viral or a heterologous promoter, followed by the R and U5 region.

According to the invention the term "polylinker" is used for a short stretch of artificially synthesized DNA which carries a number of unique restriction sites allowing the easy insertion of any promoter or DNA segment. The term "heterologous" is used for any combination of DNA sequences that is not normally found intimately associated in nature.

With reference to the procon vectors, said polylinker sequence carries at least one unique restriction site and contains preferably at least one insertion of a

heterologous DNA fragment. Said heterologous DNA fragment is preferably selected from regulatory elements and promoters, preferably being target cell specific in their expression. The retroviral promoter structure is termed LTR. LTR's carry signals that allow them to jump in and out of the genome of the target cell. Such jumping transposable elements can also contribute to pathogenic changes. Procon vectors can carry modified LTR's that no longer carry the signals required for jumping. Again this increases the potential safety of these vector systems.

Gene expression is regulated by promoters. In the absence of promoter function a gene will not be expressed. The normal MLV retroviral promoter is fairly unselective in that it is active in most cell types. However a number of promoters exist that show activity only in very specific cell types. Such tissue-specific promoters will be the ideal candidates for the regulation of gene expression in retroviral vectors, limiting expression of the therapeutic genes to specific target cells.

The target cell specific regulatory elements and promoters are preferably, but not limited to one or more elements of the group consisting of, Whey Acidic Protein (WAP), Mouse Mammary Tumour Virus (MMTV), β -lactoglobulin and casein specific regulatory elements and promoters, which may be used to target human mammary tumours, pancreas specific regulatory elements and promoters including carbonic anhydrase II and β -glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including human immunodeficiency virus (HIV), immunoglobulin and MMTV lymphocytic specific regulatory elements and promoters and MMTV specific regulatory elements and promoters such as ^{MMTV}P2 conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland, T-cell specific regulatory elements and promoters such as T-cell receptor gene and CD4 receptor promoter and B-cell specific regulatory elements and promoters such as immunoglobulin promoter or mb1 Said regulatory elements and promoters regulate preferably the expression of at least one of the coding sequences of said retroviral vector.

The LTR regions are preferably, but not limited, selected from at least one element of the group consisting of LTR's of Murine Leukaemia Virus (MLV), Mouse

Mammary Tumour Virus (MMTV), Murine Sarcoma Virus (MSV), Simian Immunodeficiency Virus (SIV), Human Immunodeficiency Virus (HIV), Human T-cell Leukaemia Virus (HTLV), Feline Immunodeficiency Virus (FIV), Feline Leukaemia Virus (FELV), Bovine Leukaemia Virus (BLV) and Mason-Pfizer-Monkey virus (MPMV).

The antimicrobial genes of the present invention will be placed under the transcriptional control of for instance the HIV promoter or a minimal promoter placed under the regulation of the HIV tat responsive element (TAR) to target HIV infected cells. Targeting will be achieved because the HIV promoter is dependent upon the presence of Tat, an HIV encoded autoregulatory protein (Haseltine, 1991). Thus only cells infected with HIV and therefore expressing Tat will be able to produce the amphipathic peptide introduced in the Procon vector (Fig. 2). Alternatively, the amphipathic peptide could be expressed from T cell specific promoters such as that from the CD4 or T cell receptor gene. In order to target tumour cells, promoters from genes known to be overexpressed in these cells (for example c-myc, c-fos) may be used.

The antimicrobial genes of the present invention may be placed also under the transcriptional control of other promoters known in the art. Examples for such promoters are of the group of SV40, cytomegalovirus, Rous sarcoma virus, β -actin, HIV-LTR, MMTV-LTR, B or T cell specific and tumour specific promoters.

The retroviral vector is in one embodiment of the invention a BAG vector (Price et al., 1987), but includes also other retroviral vectors.

According to a preferred embodiment of the invention at least one retroviral sequence encoding for a retroviral protein involved in integration of retroviruses is altered or at least partially deleted.

Said heterologous DNA fragment is preferably homologous to one or more cellular sequences. The regulatory elements and promoters are preferably regulatable by transacting molecules.

In a further embodiment of the invention a retroviral vector system is provided comprising a retroviral vector as described above as a first component and a packaging cell line harbouring at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged.

The packaging cell line harbours retroviral or recombinant retroviral constructs coding for those retroviral proteins which are not encoded in said retroviral vector. The packaging cell line is preferably selected from an element of the group consisting of ψ 2, ψ -Crip, ψ -AM, GP+E-86, PA317 and GP+envAM-12.

After replicating the retroviral vector of the invention as described above in a retroviral vector system as described above, a retroviral provirus is provided wherein said polylinker and any sequences inserted in said polylinker in the 3'LTR become duplicated during the process of reverse transcription in the infected target cell and appear in the 5'LTR as well as in the 3'LTR of the resulting provirus.

The retroviral vector of the invention refers to a DNA sequence retroviral vector on the DNA sequence level.

The invention includes, however, also a retroviral provirus and mRNA of a retroviral provirus according to the invention and any RNA resulting from a retroviral vector according to the invention and cDNAs thereof.

A further embodiment of the invention provides non-therapeutic or therapeutic method for introducing homologous and/or heterologous nucleotide sequences into human or animal cells in vitro and in vivo comprising transfecting a packaging cell line of a retroviral vector system according to the invention with a retroviral vector according to the invention and infecting a target cell population with recombinant retroviruses produced by the packaging cell line. The nucleotide sequences are

selected from one or more elements of the group consisting of genes or parts of genes encoding for therapeutic antimicrobial peptides, regulatory sequences and promoters.

The retroviral vector, the retroviral vector system and the retroviral provirus as well as RNA thereof is used for producing a pharmaceutical composition for somatic gene therapy in mammals including humans. Furthermore, they are used for targeted integration in homologous cellular sequences.

Principle for the Construction of Procon Vectors for targeted gene expression

In the murine leukemia virus (MLV) retroviral vector known as BAG (Price et al., 1987) the β -galactosidase gene is driven by the promiscuous (i.e. non-tissue specific) MLV promoter in the U3 region of the LTR (Fig. 3). According to one embodiment the present invention a derivative of the BAG vector has been constructed in which the MLV promoter (U3) located within the 3'LTR (Fig. 3) has been deleted and replaced with a polylinker, said polylinker allowing the facile introduction of heterologous promoters. The BAG vector lacking the U3 is expressed from the MLV promoter (U3) within the 5'LTR when introduced into a packaging cell line. As a result of the rearrangements occurring in the retroviral genome during its life cycle, following infection of its target cell, the polylinker will be duplicated at both ends of the retroviral genome as described above. Thereby a retroviral vector can be constructed in which the expression of the β -galactosidase gene of BAG will be controlled by the polylinker or any promoter inserted into the polylinker in the target cell (Fig. 3).

Further, the replacement of β -galactosidase with a therapeutic gene such as one encoding melittin, cecropin or another antimicrobial peptide will result in a retroviral vector that can be manipulated to express this gene from any inserted promoter.

Procon vectors carrying tissues specific promoters and regulatory elements such as the tat responsive element (TAR) from HIV will be useful for directing the

expression of the therapeutic naturally occurring antimicrobial peptid sequences or derivatives thereof to predefined cell types, tissues and organs. Potential therapeutic sequences include melittin, which has anti-HIV and anti-tumour effects, cecropin and megainin sequences and sequences which prime cells for death including the thymidine kinase, guanine phosphoribosyltransferase and cytosine deaminase genes.

The Drawings

- Figure 1** The mode of reverse transcription of a retrovirus.
- Figure 2** Retroviral vector constructs carrying melittin and cecropin coding sequences.
- Figure 3** Construction of U3 minus BAG-vector (MLV).
- Figure 4** The construction of the retroviral vector p125.Cecr.A carrying the PreProCecropin A gene.
- Figure 5** The construction of the retroviral vectors pBAGpMel and pBAGppMel carrying respectively the PreMelittin and the PreProMelittin gene.
- Figure 6** pBAGpMel and pBAGppMel
- Figure 7** pBAG
- Figure 8** Principle of S1 analysis of transfected Cecropin and Melittin clones.
- Figure 9** S1 analysis: PAGE gel electrophoresis and Phospho-imaging exposition.
- Figure 10** Antitumour activity of retroviral vectors carrying PreProCecropin coding sequences.

- Figure 11 Antitumour activity of retroviral vectors carrying Melittin coding sequences.
- Figure 12 Downregulation of expression from HIV LTR of retroviral vectors carrying Cecropin and Melittin coding sequences.
- Figure 13 Downregulation of expression of HIV LTR of retroviral vectors carrying Cecropin coding sequences.
- Figure 14 Downregulation of LTR's from Mouse Mammary Tumour Virus (MMTV) by retroviral vectors carrying Cecropin and Melittin coding sequences.

The following examples will illustrate the invention further. These examples are however in no way intended to limit the scope of the present invention as obvious modifications will be apparent, and still other modifications and substitutions will be apparent to anyone skilled in the art.

The recombinant DNA methods employed in practicing the present invention are standard procedures, well known to those skilled in the art, and described in detail, for example, in Molecular Cloning, Sambrook, et al., Cold Spring Harbor Laboratory, (1989) and B. Perbal, A Practical Guide to Molecular Cloning, John Wiley & Sons (1984).

EXAMPLES

Construction of retroviral vectors carrying the PreProCecropin A sequence

Construction of p125gal

In the murine leukemia virus (MLV) retroviral vector known as BAG (Price et., 1987) the β -galactosidase gene is driven by the promiscuous (i.e. non-tissue specific) MLV promoter in the U3 region of the LTR (Fig. 3). A derivative of the BAG vector has been constructed in which the MLV promoter (U3) located within the 3'LTR (Fig. 3) has been deleted by PCR. At this position a polylinker was inserted containing the restriction sites *Sac*II and *Mlu*I allowing the facile introduction of heterologous promoters. The BAG vector lacking the U3 is expressed from the MLV promoter (U3) within the 5'LTR when introduced into a packaging cell line. As a result of the rearrangements occurring in the retroviral genome during its life cycle, following infection of its target cell, the polylinker will be duplicated at both ends of the retroviral genome as described in danish patent application no. 1017/94. Thereby a retroviral vector can be constructed in which the expression of the β -galactosidase gene of BAG will be controlled by the polylinker or any promoter inserted into the polylinker in the target cell (Fig. 3).

The Mouse Mammary Tumour Virus (MMTV) U3-region (mtv-2) without the inverted repeats, which contains the MMTV promoters as well as a region that confers responsiveness to glucocorticoid hormones and a region containing an element that directs expression to the mammary gland was inserted into the polylinker region of the modified BAG vector to produce p125gal (fig. 4a).

Construction of p125CecrA

Figure 4 illustrates the construction of the retroviral vector p125CecrA. The plasmid pCecrA (obtained from Boman et al.) and illustrated in fig. 4a carries the cDNA of the PreProCecropin A gene from the giant silk moth (*Hyaophora cecropia*) cloned in front of the bacterial sp6 promoter. The PreProCecropin sequence was obtained by amplification by PCR from this plasmid using the following primers:

Primer 1: CecrA1 5'-TATGACGTC-TCGTTAGAACGCGGCT-3'

Primer 2: CecrA3 5'-GGCAGATCT-TAAATGTATCATGCAAT-3'

CecrA1 carries an *Aat*II restriction site (GACGTC) in the 5' extension with a 3 base pair protection (TAT). Similarly CecrA3 carries a *Bgl*II site (AGATCT) in the 5' extension with a 3 base pair protection (GGC).

The PCR product (370 bps) was then digested with *Aat*II and *Bgl*II to make the restriction sites available for cloning (Fig 4b).

P125gal was digested with the restriction enzymes *Aat*II and *Bam*HI resulting in a fragment of 5043 base pairs (Fig 4b) and ligated to the fragment carrying the PreProCecropin A gene from pCecrA. This resulted in the formation of p125.CecrA (Fig 4c), the *Bam*HI/*Bgl*II sites being lost.

Construction of retroviral vectors carrying the PrePreMelittin and PreMelittin sequence

Figure 5 illustrates the construction of the retroviral vectors pBAGpMel and pBAGppMel. The plasmid pUM13/4 (Vlasak, R., Uger-Ullmann, C., Keil, G. and Frischaut, A-M, Nucleotide sequences of cloned cDNA coding for honeybee prepromelittin, Eur. J. Biochem. 135:123-126 (1989)) (Fig 5a) carries the cDNA encoding the PrePromelittin gene from *apis mellifera*. The PrePromelittin and the

Premelittin sequence was obtained by amplification by PCR using the following primers:

Primer 1: 5'-ATAGACGTC-AAGGAAGGAAGCGATCGGA-3'
Primer 2: 5'-TATGGATCC-AACCCCTGTTGCCTCTTACG-3'
Primer 3: 5'-TCTTACATCTATGCG-GGAATTGGAGCAGTTCTGAA-3'
Primer 3': 5'-AACTGCTCCAATTCC-CGCATAGATGTAAGAAATGT-3'

Primer 1 carries an *Aat* II site (GACGTC) in the 5' extension with an ATA 3 bp protection and primer 2 carries a *Bam* HI site (GGATCC) in the 5' extension with a TAT 3 bp protection.

A PCR on the plasmid pUM13/4 with primers 1 and 2 results in the amplification of the whole PreProMelittin sequence (Fig 5 bi) carrying both *Aat* II and *Bam* HI sites to enable cloning into the retroviral vector pBAG (Fig 5a) after digestion of the plasmid with *Aat* II and *Bam* HI (Fig 5c). The resulting plasmid pBAGppMel is shown in Fig 6.

The amplification of the PreMelittin sequence was carried out using a combination of conventional PCR and recombinant PCR. Primer 3, though binding in the melittin gene, carries a 5' extension that corresponds to the 3' sequence of the Pre sequence. Similarly, the Primer 3', though binding in the 3' region of the Pre sequence, carries a 5' extension corresponding to the sequence in the 5' region of the melittin gene. Initially a PCR was made with either primer pair 1 and 3 or 2 and 3. The products from these two reactions were then used to make a recombinant PCR. This entailed hybridising the two PCR products with each other using the 5' extensions that were carried in the primers 3 and 3'. Subsequent addition of primers 1 and 2, which adds the *Aat* II and *Bam* HI sites for cloning into pBAG allowed the amplification of the entire PreMelittin sequence (Fig 5 bii). Plasmid pBAGpMel (Fig 6) was produced by digestion of this PCR product and pBAG with *Aat* II and *Bam* HI followed by ligation of the fragments.

Isolation of clones

Ej cells was tranfected with p125.CercA and the clones expressing neomycine resistance was isolated. Six clones was isolated: Cecropin A1.4, Cecropin A1.7, Cecropin A1.8, Cecropin A10.3, Cecropin A10.4, and Cecropin A10.8.

Similarly Ej cells was tranfected with pBAGpMel and pBAGppMel and the clones expressing neomycine resistance was isolated. Three clones containing the premelittin gene was isolated: Pre Melittin 1, Pre Melittin 4, and Pre Melittin 6. Two clones containing prepromelittin was isolated: Pre Pro Melittin 1, and Pre Pro Melittin 5.

S1 analysis of transfected Cecropin/Melittin clones

An S1 analysis was performed using pBAG digested with SpeI as a probe (Fig 7), the resulting 6.1 kb fragment comprising 3'LTR, polyoma early region and 5'LTR. The fragment was then end-labelled using polynucleotide kinase with 32 P and hybridised against total RNA isolated from EJ (human bladder carcinoma cell line) cell clones that had been transfected with retriviral vector constructs bearing either preprocecropin (clones A1.4, A1.7, A1.8, A10.4, A10.3, and A10.8), premelittin (clones 1, 4 and 6), and prepromelittin (clones 1 and 5) or the empty vector alone (pBAG clones 3 and 6) (Fig 8a).

There followed an S1 nuclease digestion (Fig 8b), the enzyme cleaving only single stranded necleic acids, resulting in a 288 bp fragment arising from the hybridisation of the probe to the mRNA coming from the retroviral vector that is present in the genomic DNA of the transfected clones.

Subcequent separation using a 6% polyacrylamide gel and exposure of the gel using a Phosphor-imaging system (Fuji BAS1000) revealed the expected bands as can be seen in figure 9.

Antitumour experiments

Many amphipathic polypeptides are synthesized in a preproform which is inactive (Boman, 1995). The endopeptidase that cleaves of the presignal peptide in a co-translational process is thought to be present in all cells whereas the protease that converts promelittin to the active melittin form appears to be present only in certain cells (Krell et al., 1980).

The human bladder carcinoma derived cell line, EJ, gives tumours upon injection into immuno compromised nude mice which grow progressively larger (Fig. 10). The stable clones of EJ cells carrying the melittin or cecropin expression constructs obtained as described above was tested for their tumorigenicity in nude mice. Generally, cell clones carrying the cecropin, prepromelittin, or premelittin genes show a reduced rate of tumour growth in mice (Fig. 10-11), i.e both melittin and cecropin have anti-tumour effects. In a separate experiment (data not shown) the Cecropin A1.4 clone was tested in 4 mice, only of which showed a tumour.

Anti-viral activity

EJ derived cell clones carrying the melittin or cecropin expression vector or the parental BAG vector, not carrying a therapeutic gene, were supertransfected with an indicator construct carrying the HIV LTR (and thus the HIV promoter) linked to a firefly luciferase reporter gene (HIV-luc) in the absence or presence of a separate construct expressing Tat. In the absence of Tat there was little luciferase activity detectable from the HIV-luc construct in all cell clones as expected since the HIV promoter requires Tat for its activity. In contrast, in the presence of Tat, cell clones carrying BAG or a premelittin carrying construct show significant levels of luciferase expression, whereas a cell clone transfected with either a prepromelittin or a cecropin expression construct showed little luciferase expression (Fig. 12 and 13). This suggests that cecropin, prepromelittin and to a lesser extent premelittin inhibit the Tat driven expression from the HIV LTR. Thus the production of HIV from infected cells will be inhibited by the antimicrobial peptide gene carrying therapeutic

retroviral vector. This effect is expected to lead to a lack of virus production from HIV infected cells.

A similar experiment to that of figure 12 and 13 can be seen in figure 14 whereby two different MMTV LTR's controlling the expression of a luciferase reporter gene (Wintersberger et al., Proc. Natl. Acad. Sci. USA 92, 2745-2749) were separately supertransfected into cells carrying either PreProCecropin or PrePro Melittin under control of an MLV promoter. These experiments showed that the downregulatory effect of Cecropin is not restricted to the HIV promoter, but also work with another retroviral vector.

In conclusion the present invention provides therapeutic products for the treatment of retroviral infections including HIV, tumours, bacterial and viral infections comprising vector constructs carrying genes or derivatives thereof of therapeutic active peptides including those for melittins, cecropins and magainins.

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CLAIMS

1. A recombinant vector for introducing into an eucaryotic cell DNA, the vector comprising, in operable linkage,
 - a) the DNA of or corresponding to at least a portion of a retroviral vector, which portion is capable of infecting and directing the expression in the target cells; and
 - b) one or more coding sequences wherein at least one sequence encodes for at least one naturally occurring therapeutic antimicrobial peptide or a derivative thereof for the treatment of at least one disease, selected from mammalian tumours, viral, bacterial and fungal infections.
2. The recombinant vector according to claim 1, wherein said retroviral vector is replication-defective.
3. The recombinant vector according to claim 1, wherein said retrovirus vector includes, in operable linkage, a 5' LTR region and a 3' LTR region, said 5' LTR region comprising the structure U3-R-U5 and said 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by one or more of said coding sequences wherein at least one sequence encodes for at least one naturally occurring therapeutic antimicrobial peptide or a derivative thereof for the treatment of at least one disease, selected from mammalian tumours, viral, bacterial and fungal infections, followed by the R and U5 region.
4. The recombinant vector according to claim 1, wherein said retroviral vector includes, in operable linkage, a 5'LTR region; one or more of said coding sequences wherein at least one sequence encodes for at least one naturally occurring therapeutic antimicrobial peptide or a derivative thereof for the treatment of at least one disease, selected from mammalian tumours, viral, bacterial and fungal infections; and a 3' LTR region; said 5'LTR region comprising the structure U3-R-U5 and said 3' LTR region comprising a completely or partially deleted U3 region wherein said deleted U3 region is replaced by a polylinker sequence, followed by the R and U5 region to undergo promoter conversion.

5. The recombinant vector according to claim 1-4 where in said sequence coding a naturally occurring therapeutic antimicrobial peptide or derivative thereof encodes for the amino acid sequence of melittin, cecropin, magainin, apidaecin and defensin peptides or a part, an analogue, a homologue, a recombinant or a combination thereof of such antimicrobial peptides.
6. The recombinant vector according to claim 5 wherein the structural analogues of melittin include an amphiphilic helix with or without signal peptide and activation domains.
7. The recombinant vector according to claim 5 wherein the structural analogue of melittin is Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Gly-Gly-Gly-Gly-Gly.
8. The recombinant vector according to claim 5 wherein said structural analogue is Amfi 1 and peptides of GP41 that are melittin-like.
9. The recombinant vector according to claim 5 wherein said structural analogue is Amfi 2 and peptides of GP41 that are melittin-like.
10. The recombinant vector according to claim 5 wherein said melittin gene sequence or a derivative thereof is a melittin, a premelittin, or a prepromelittin encoding sequence.
11. The recombinant vector according to claim 5 wherein said sequence encoding a naturally occurring antimicrobial peptide or a derivative thereof is a cecropin, prececropin, or preprocecropin encoding sequence.
12. The recombinant vector according to claim 5 wherein said sequence encoding a naturally occurring antimicrobial peptide or a derivative thereof is a magainin encoding sequence or a derivative thereof.

13. The recombinant vector according to claim 4, wherein said polylinker sequence carries at least one unique restriction site.
14. The recombinant vector according to claim 4, wherein said polylinker sequence contains at least one insertion of a heterologous DNA fragment.
15. The recombinant vector according to claim 14, wherein said heterologous DNA fragment is selected from one or more elements of the group consisting of regulatory elements and promoters.
16. The recombinant vector according to claims 1-4, wherein said coding sequence comprises additionally at least one non-coding sequence selected from regulatory elements and promoters.
17. The recombinant vector according to claim 16, wherein said regulatory elements or promoters is selected from the group of SV40, cytomegalovirus, Rous sarcoma virus, β -actin, HIV-LTR, MMTV-LTR, B or T cell specific and tumour specific promoters.
18. The recombinant vector according to claims 15-16, wherein said regulatory elements and promoters are target cell specific in their expression.
19. The recombinant vector according to claim 18, wherein said target cell specific regulatory elements and promoters are selected from one or more elements of the group consisting of HIV, WAP, MMTV, β -lactoglobulin and casein specific regulatory elements and promoters, pancreas specific regulatory elements and promoters including carbonic anhydrase II and β -glucokinase regulatory elements and promoters, lymphocyte specific regulatory elements and promoters including immunoglobulin and MMTV lymphocytic specific regulatory elements and promoters and MMTV specific regulatory elements and promoters such as ^{MMTV}P2 conferring responsiveness to glucocorticoid hormones or directing expression to the mammary gland, T-cell specific regulatory elements and promoters such as the

T-cell receptor gene, the CD4 receptor promoter, and B-cell specific regulatory elements and promoters such as the immunoglobulin promoter and mb1.

20. The recombinant vector according to claim 15-16, wherein said regulatory elements are regulatable by transacting molecules.

21. The recombinant vector according to claims 15-16, wherein said regulatory elements and promoters regulate the expression of at least one of the coding sequences of said retroviral vector.

22. The recombinant vector according to claim 1-4, wherein the LTR regions of said retroviral vector are selected from at least one element of the group consisting of LTRs of MLV, MMTV, MSV, SIV, HIV, HTLV, FIV, FeLV, BLV and MPMV.

23. The recombinant vector according to claim 22, wherein said retroviral vector is a BAG vector.

24. The recombinant vector according to claim 1-4, wherein said coding sequence is additionally selected from one or more elements of the group consisting of marker genes, therapeutic genes, antiviral genes, antitumour genes, cytokine genes.

25. The recombinant vector according to claim 24, wherein said marker or therapeutic gene is selected from the group consisting of marker genes which codes for proteins such as β -galactosidase, neomycin, alcohol dehydrogenase, puromycin, hypoxanthine phosphoribosyl transferase (HPRT), hygromycin and secreted alkaline phosphatase or therapeutic genes which codes for proteins such as Herpes Simplex Virus thymidine kinase, cytosine deaminase, guanine phosphoribosyl transferase (gpt), cytochrome P 450 and cell cycle regulatory genes which codes for proteins such as P.T.O., SDI or tumor suppressor genes which codes for proteins such as p53 or antiproliferation genes or cytokines such as IL-2.

26. The recombinant vector according to claims 1-4, wherein retroviral sequences involved in integration of retroviruses are altered or at least partially deleted.
27. The recombinant vector according to claims 1-4, wherein said retroviral vector further comprises DNA sequences homologous to one or more cellular sequences.
28. A recombinant retroviral vector system comprising a retroviral vector according to anyone of the preceding claims 1-27 as a first component; and a packaging cell line harbouring at least one retroviral or recombinant retroviral construct coding for proteins required for said retroviral vector to be packaged.
29. A non-therapeutical or therapeutical method for introducing homologous or heterologous nucleotide sequences into human or animal cells in vitro and in vivo comprising transfecting a packaging cell line of a retroviral vector system according to claim 28 with a retroviral vector according to anyone of claims 1-27, and infecting a target cell population with said recombinant retroviruses produced by the packaging cell line.
30. A retroviral provirus produced by replicating the retroviral vector of anyone of claims 1 to 27 in a retroviral vector system according to claim 28 wherein the U3 or said polylinker and any sequences inserted in said polylinker in the 3'LTR become duplicated during the process of reverse transcription in the infected target cell and appear in the 5'LTR as well as in the 3'LTR of the resulting provirus and the U5 of the 5'LTR become duplicated during the process of reverse transcription in the infected target cell and appear in the 3'LTR as well as in the 5'LTR of the resulting provirus.
31. A retroviral particle produced by transfecting a packaging cell line of a retroviral vector system according to claim 28 with a retroviral vector according to anyone of claims 1-27, and isolating said particle.

32. Use of a recombinant vector according to anyone of claims 1 to 27 for producing a pharmaceutical composition for gene therapy of at least one disease, selected from tumours, viral, bacterial and fungal infections in mammals including humans.
33. Use of a recombinant retroviral vector system according to claim 28 for producing a pharmaceutical composition for gene therapy of at least one disease, selected from tumours, viral, bacterial and fungal infections in mammals including humans.
34. Use of a retroviral particle according to claim 31 for producing a pharmaceutical composition for gene therapy of at least one disease, selected from tumours, viral, bacterial and fungal infections in mammals including humans.
35. Use of a retroviral particle according to claim 31 for targeted integration in said homologous cellular sequences.
36. mRNA of a retroviral provirus according to claim 30.
37. RNA of a vector according to anyone of claims 1 to 27.
38. A host cell infected with a virion according to claim 31.
39. A host cell according to claim 38 expressing said antimicrobial therapeutic peptide.
40. Use of cecropin, magainins or derivatives thereof for the manufacture of a medicament for treating viral infections.
41. Use according to claim 40 for the manufacture of a medicament for treating retroviral infections.

42. Use according to claim 41 for the manufacture of a medicament for treating HIV infections.

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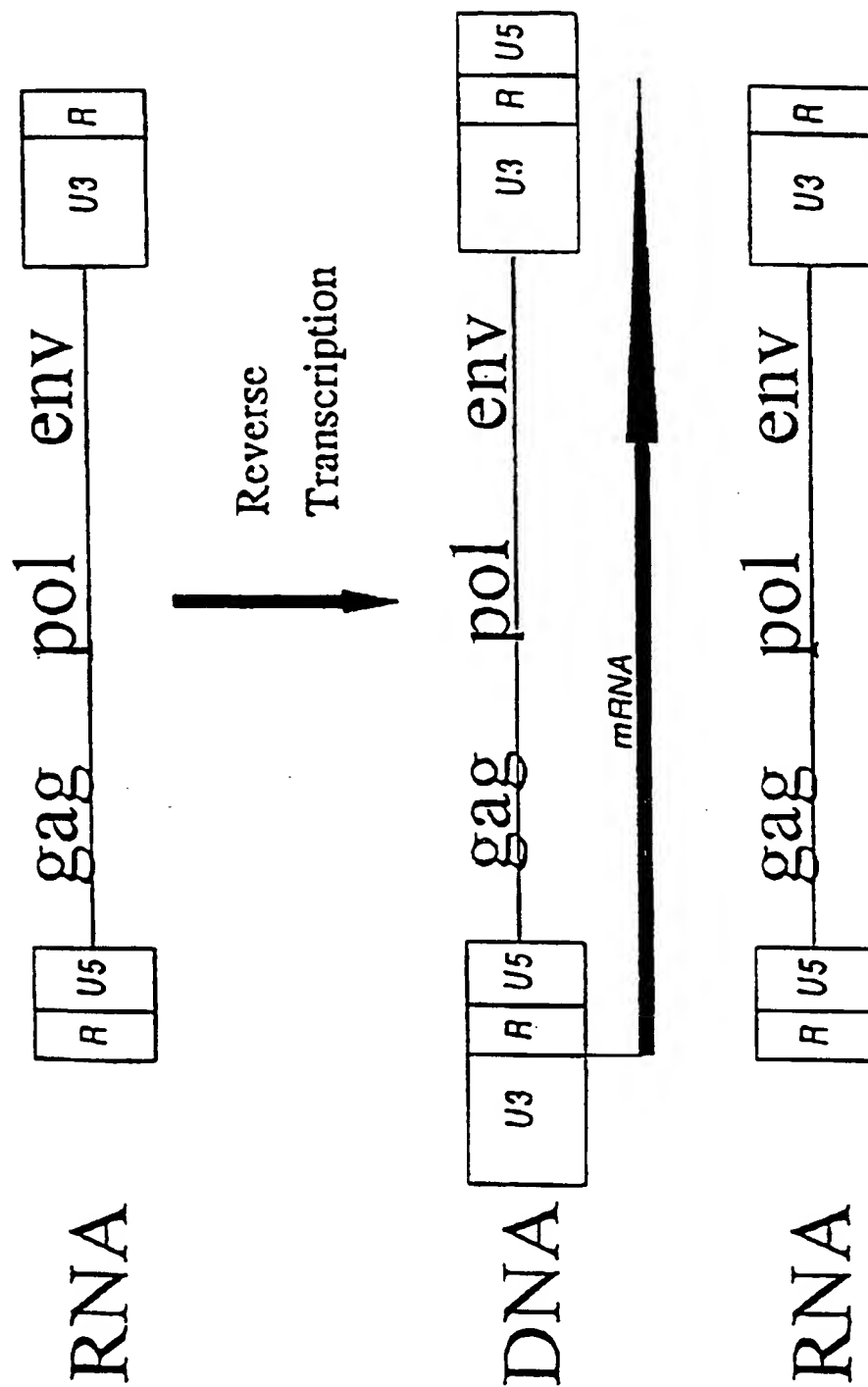


Fig. 1

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The Melittin & Cecropin Retroviral Vector Constructs

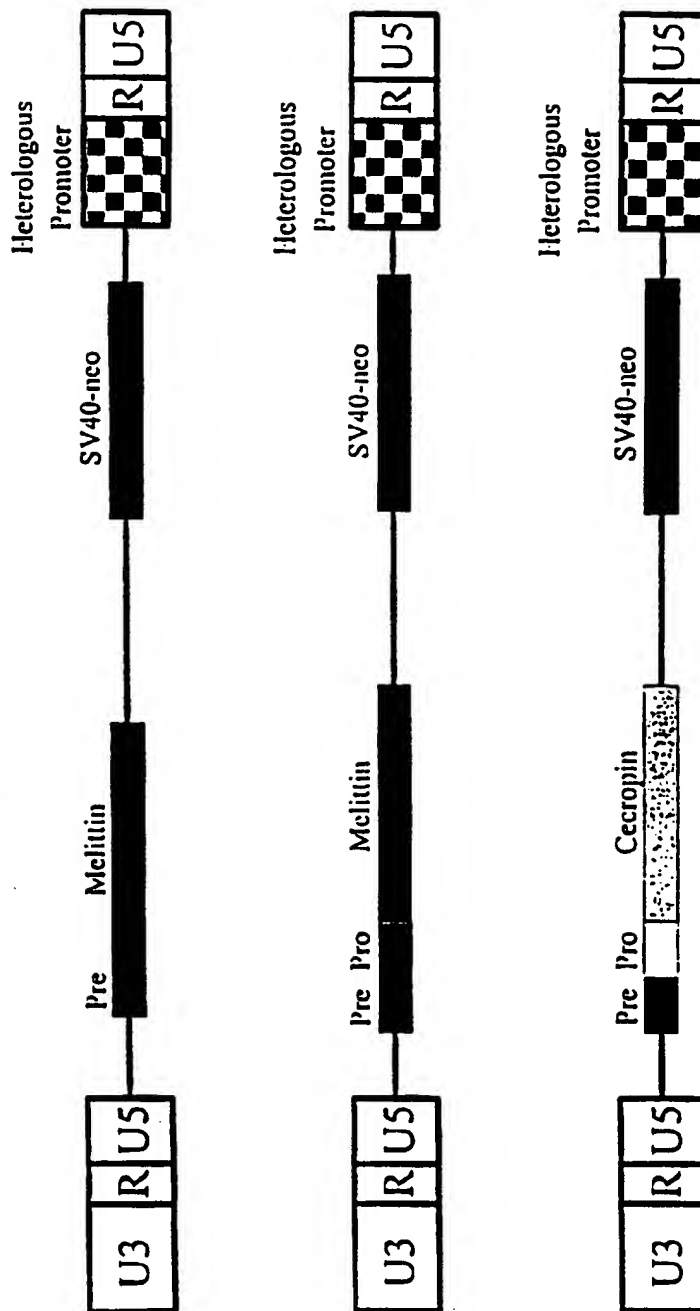


Fig. 2

Construction of a U3 minus BAG-vector (MLV)

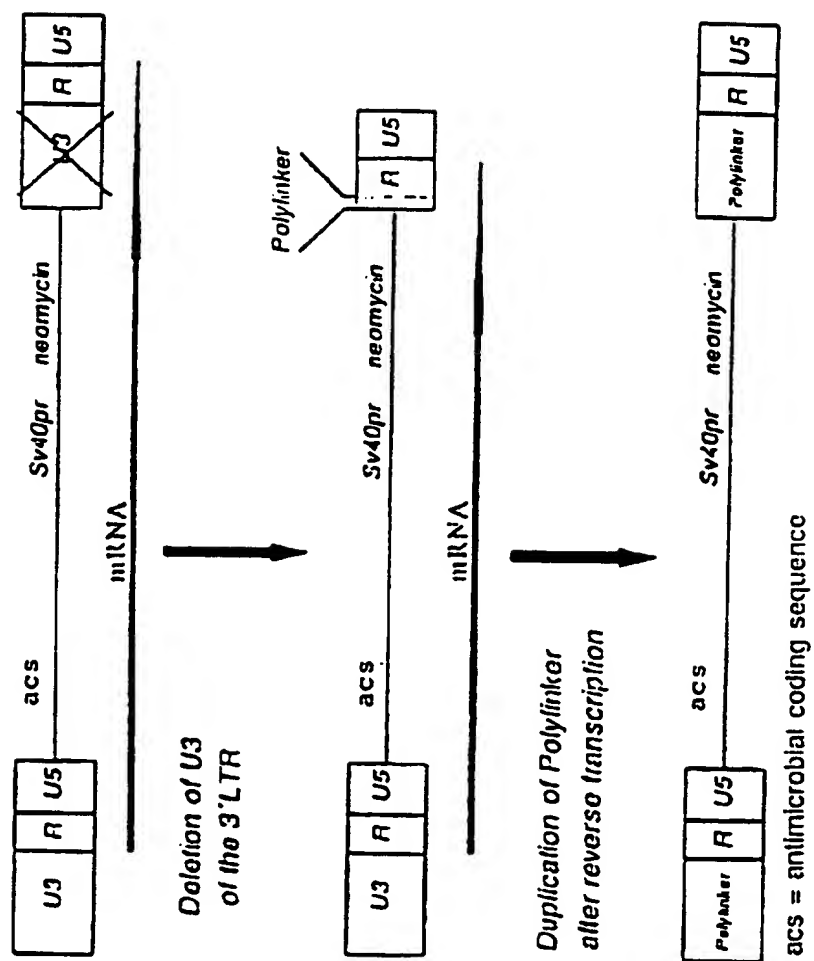


Fig. 3

acs = antimicrobial coding sequence

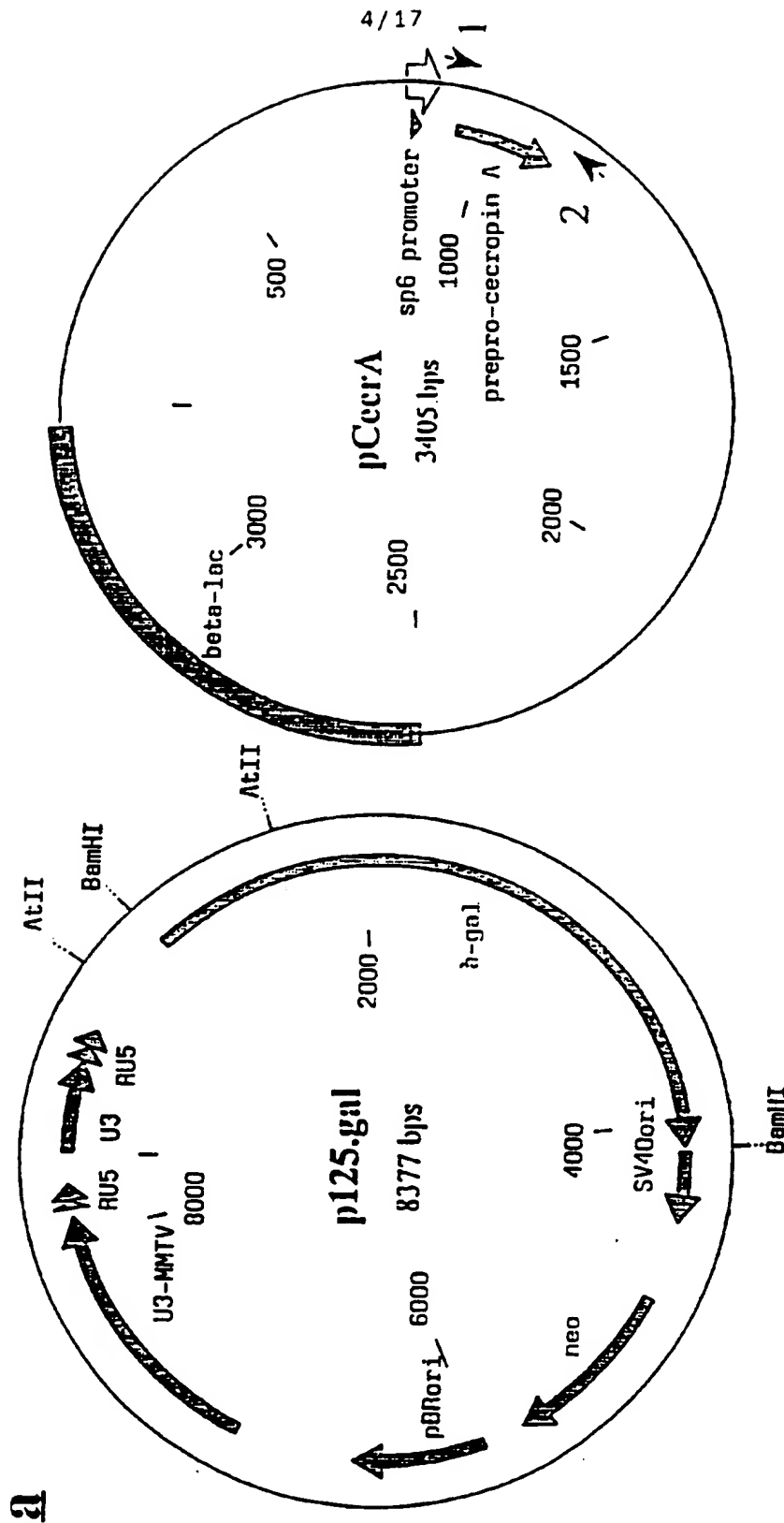


FIG 4 a

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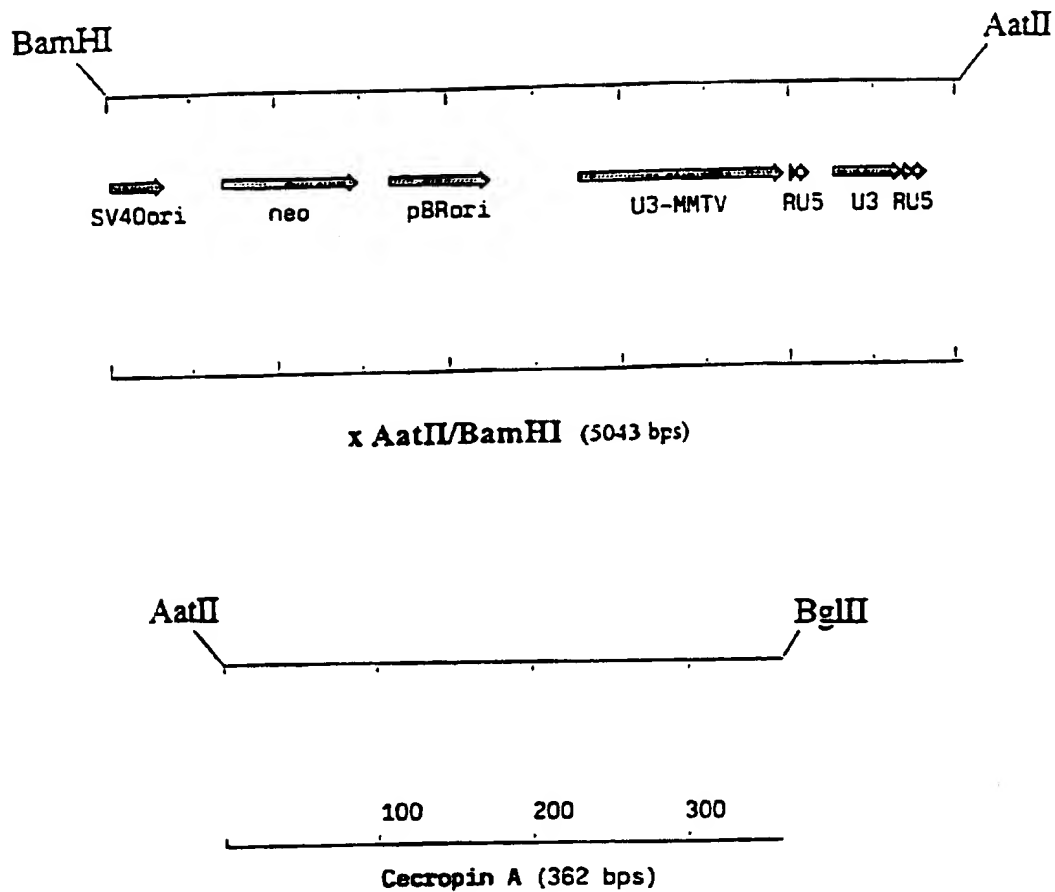
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FIG 4 b

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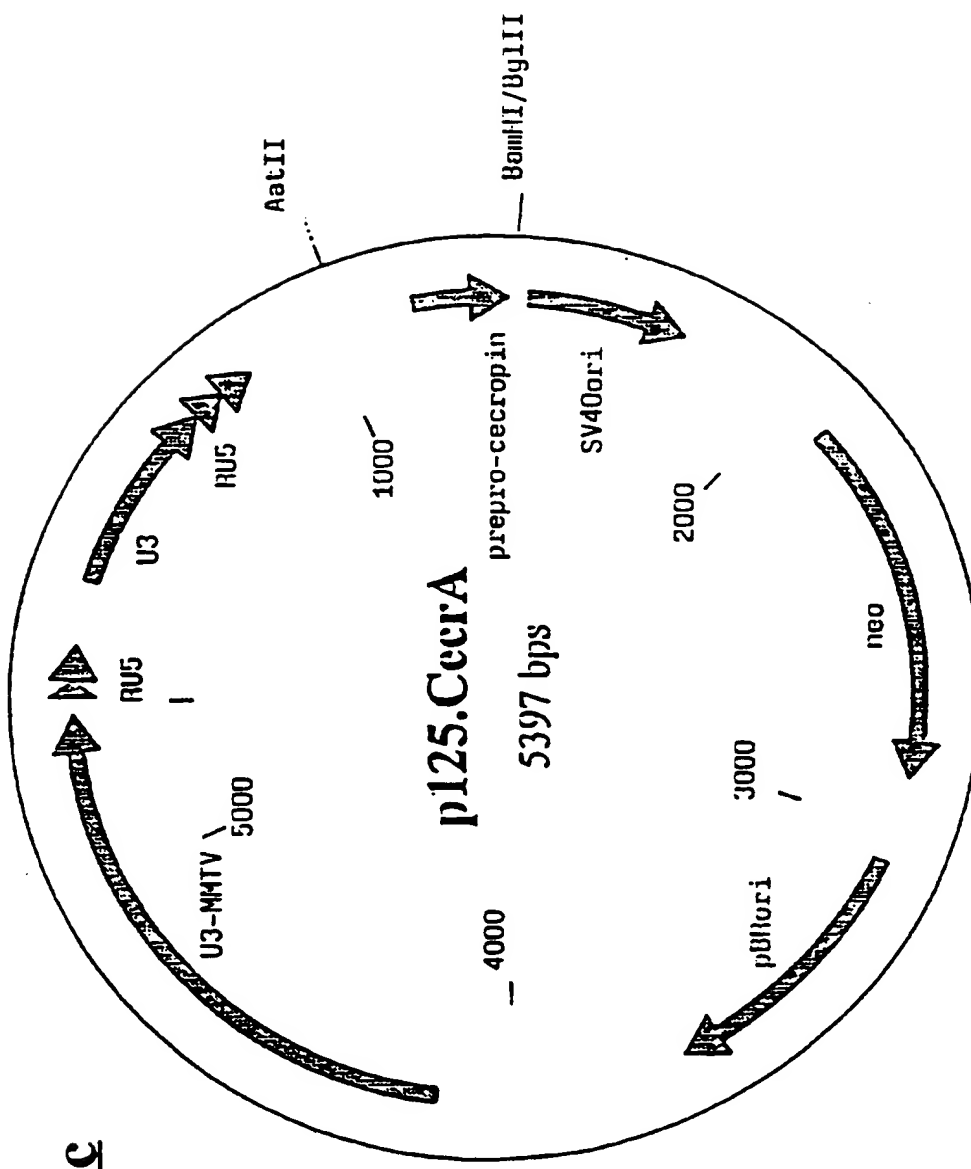


FIG 4 c

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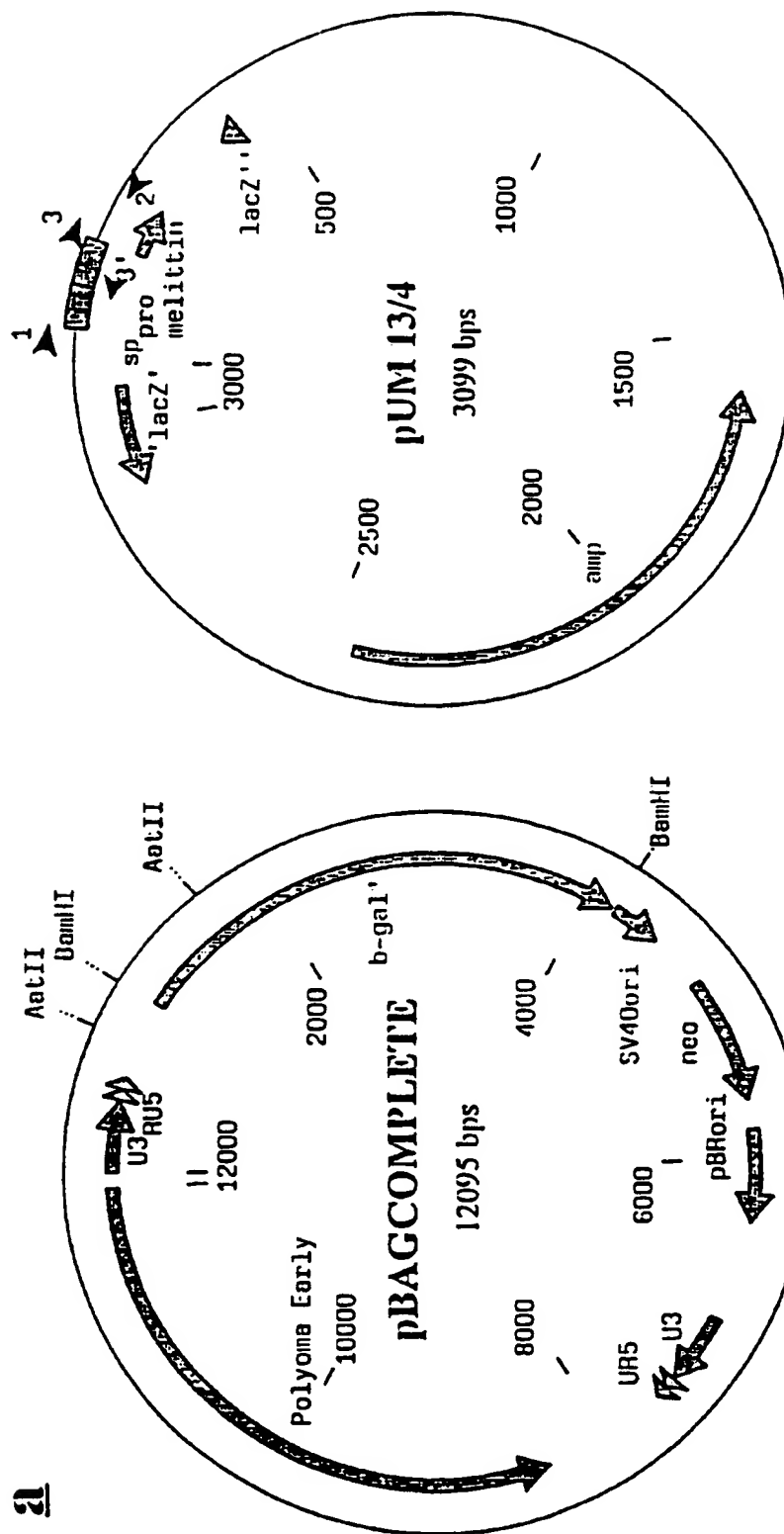


FIG 5 a

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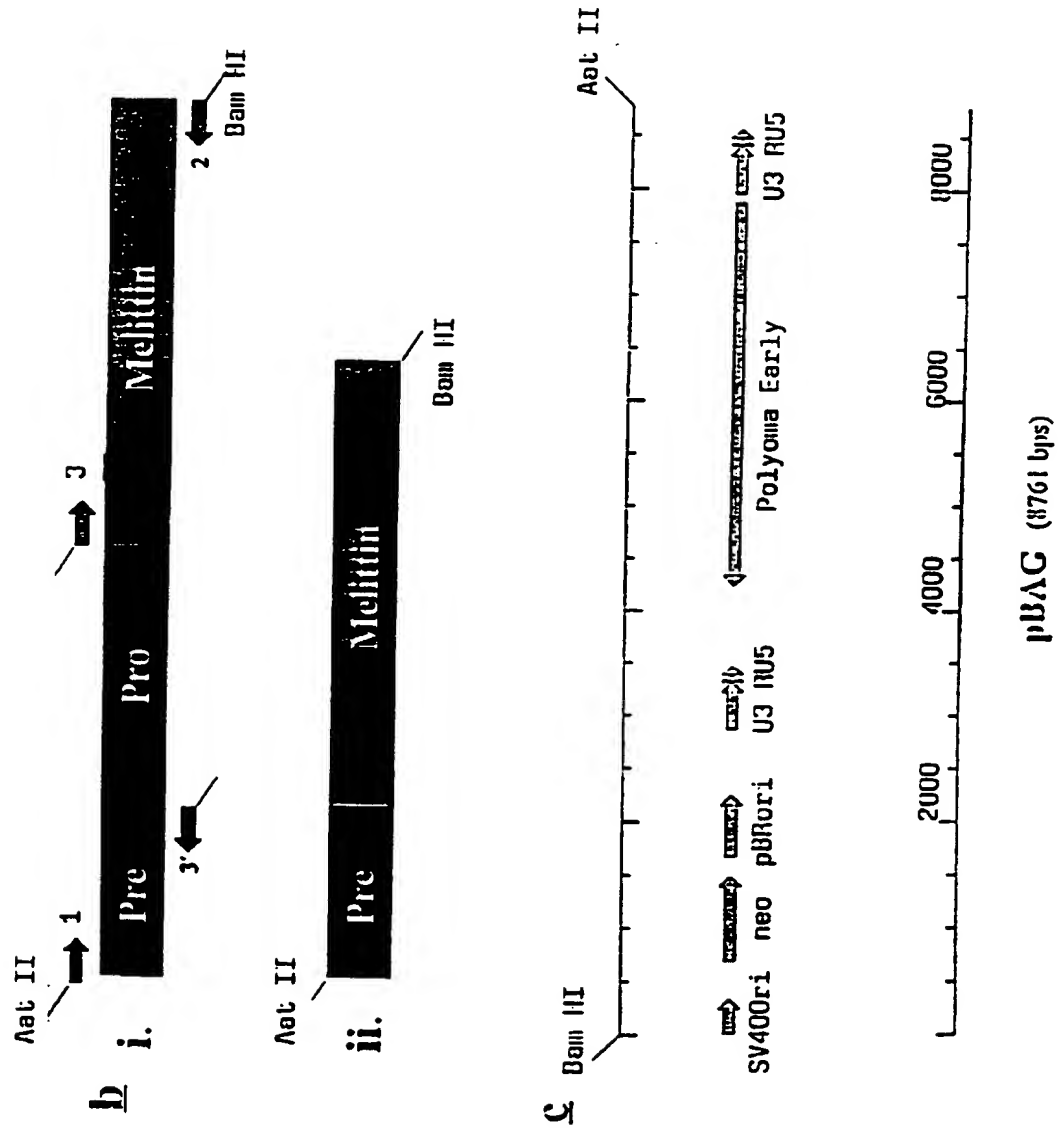


FIG 5 b-c

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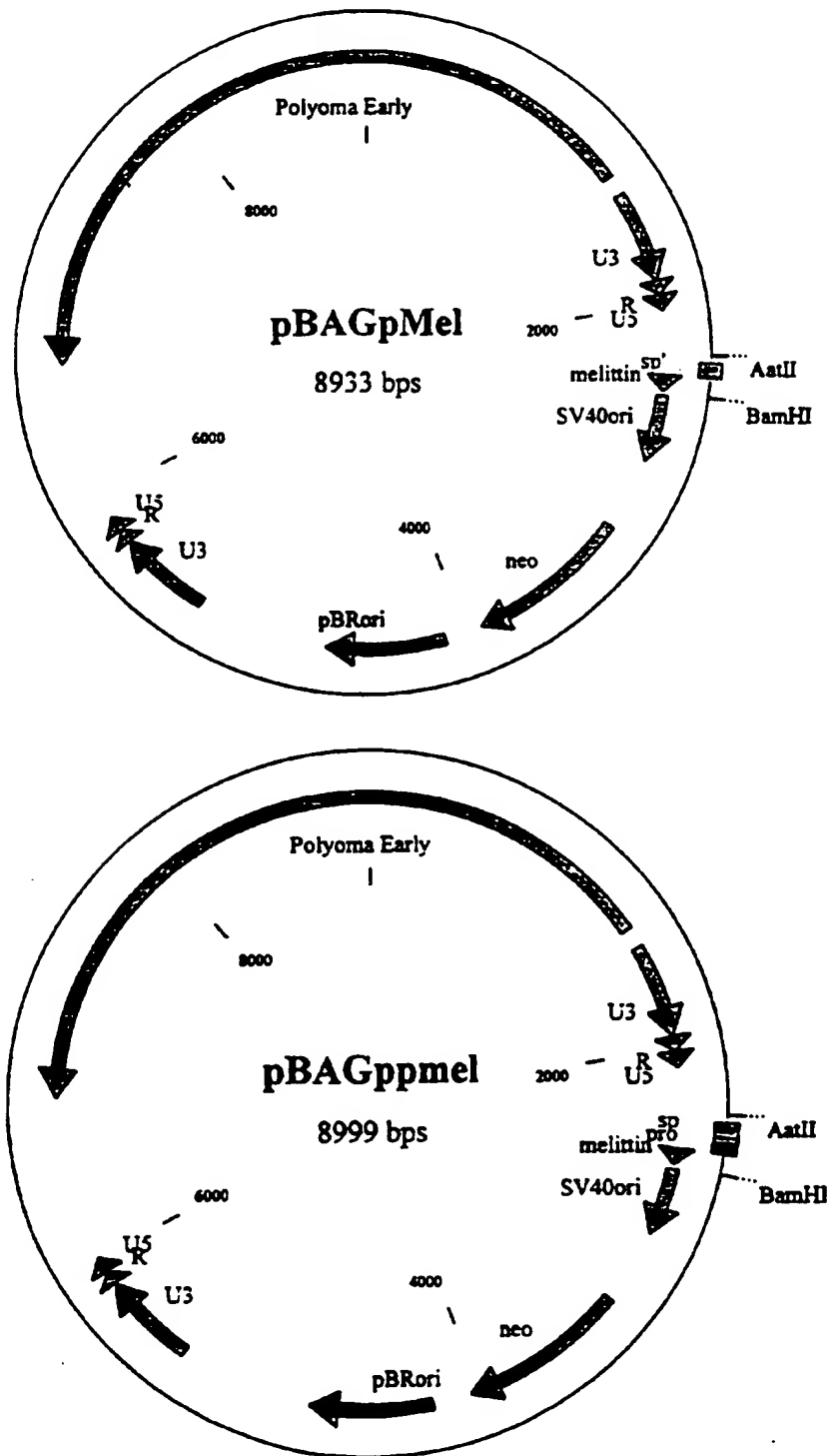


FIG 6

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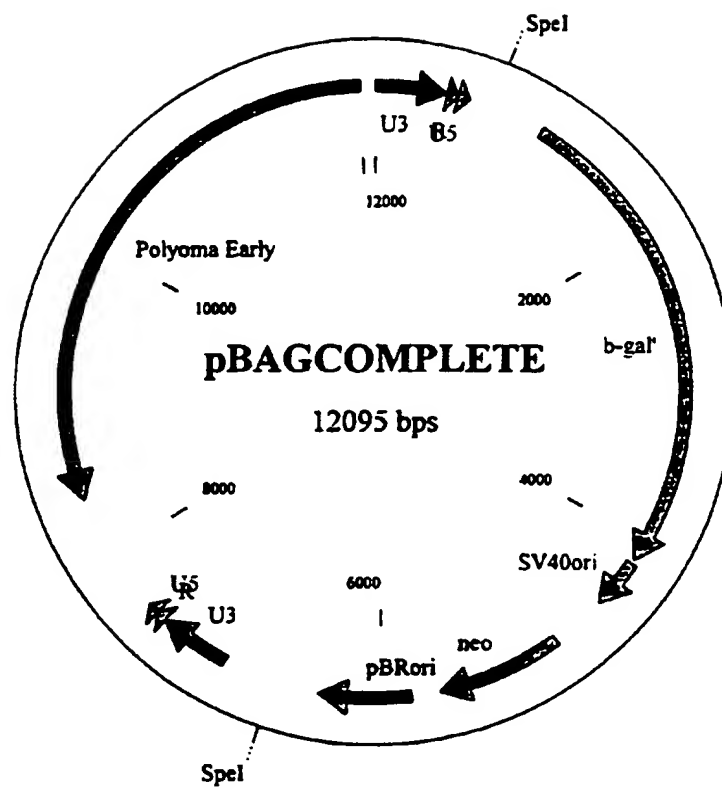
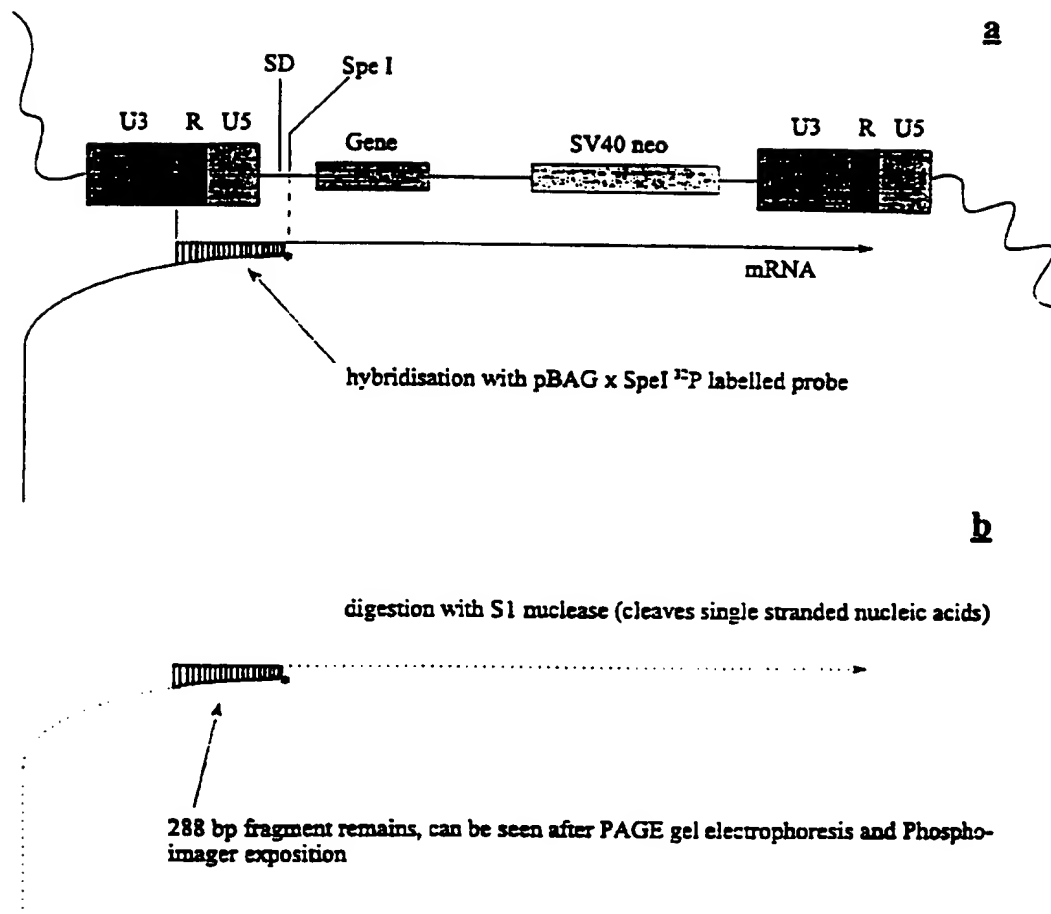


FIG 7

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**FIG 8**

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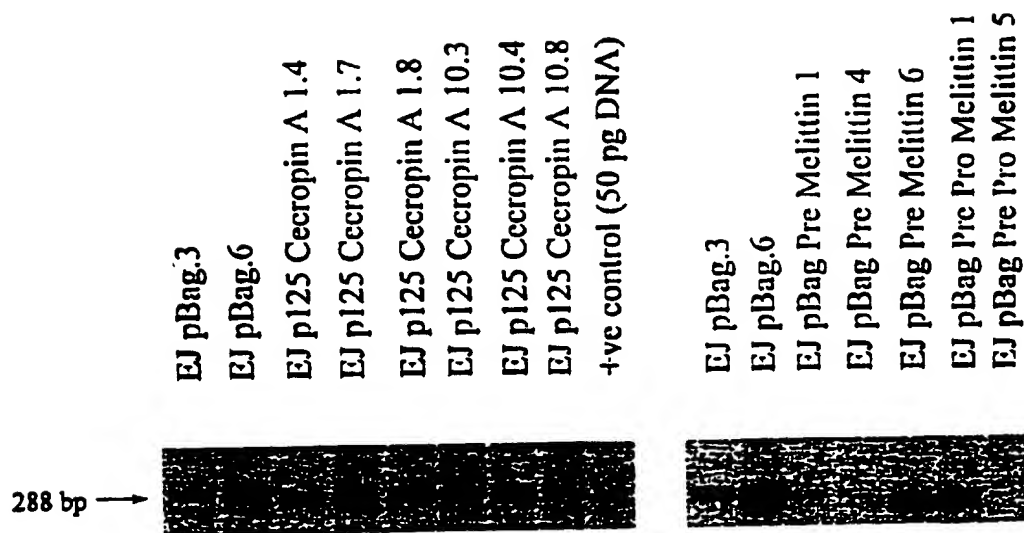


FIG 9

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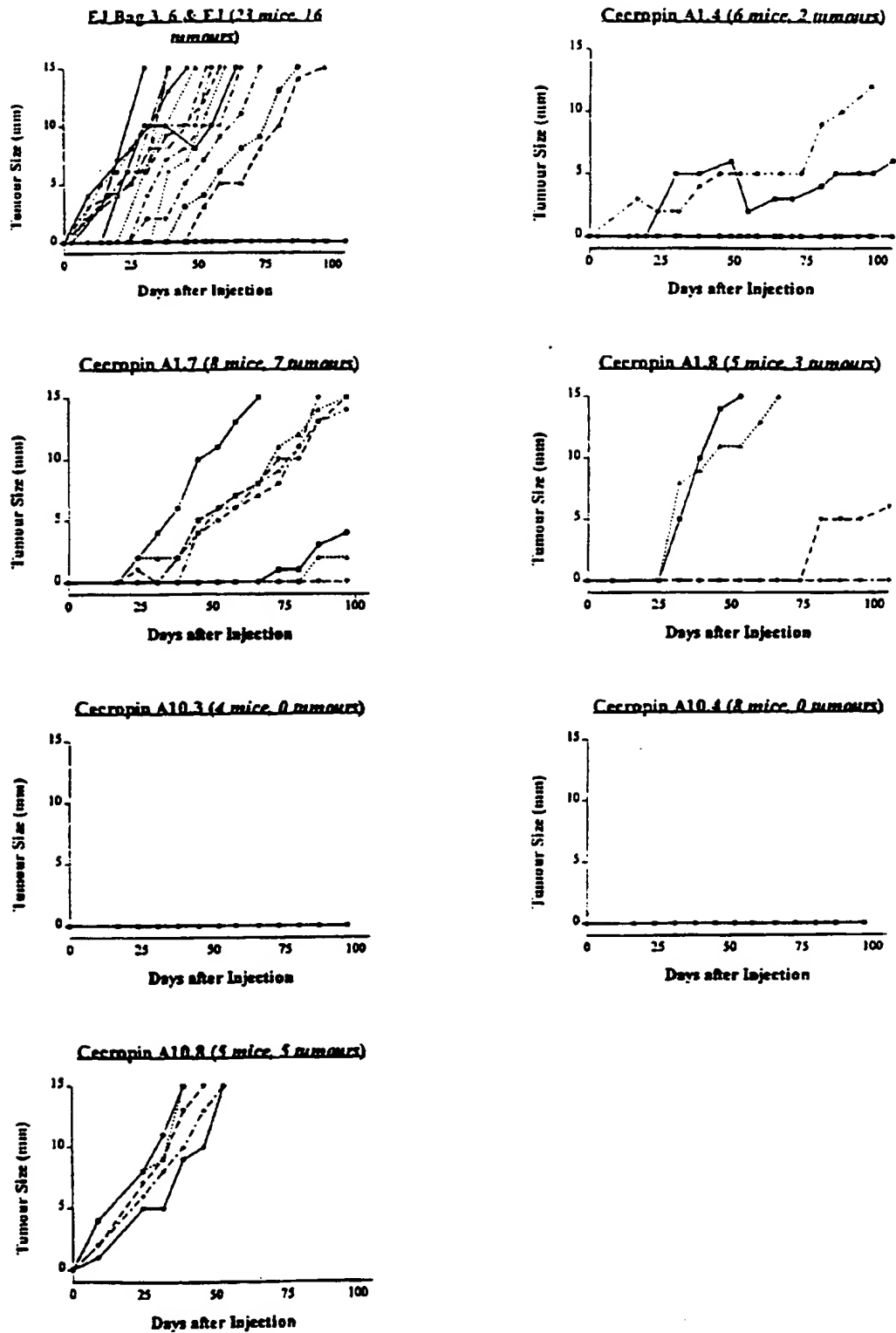


FIG 10

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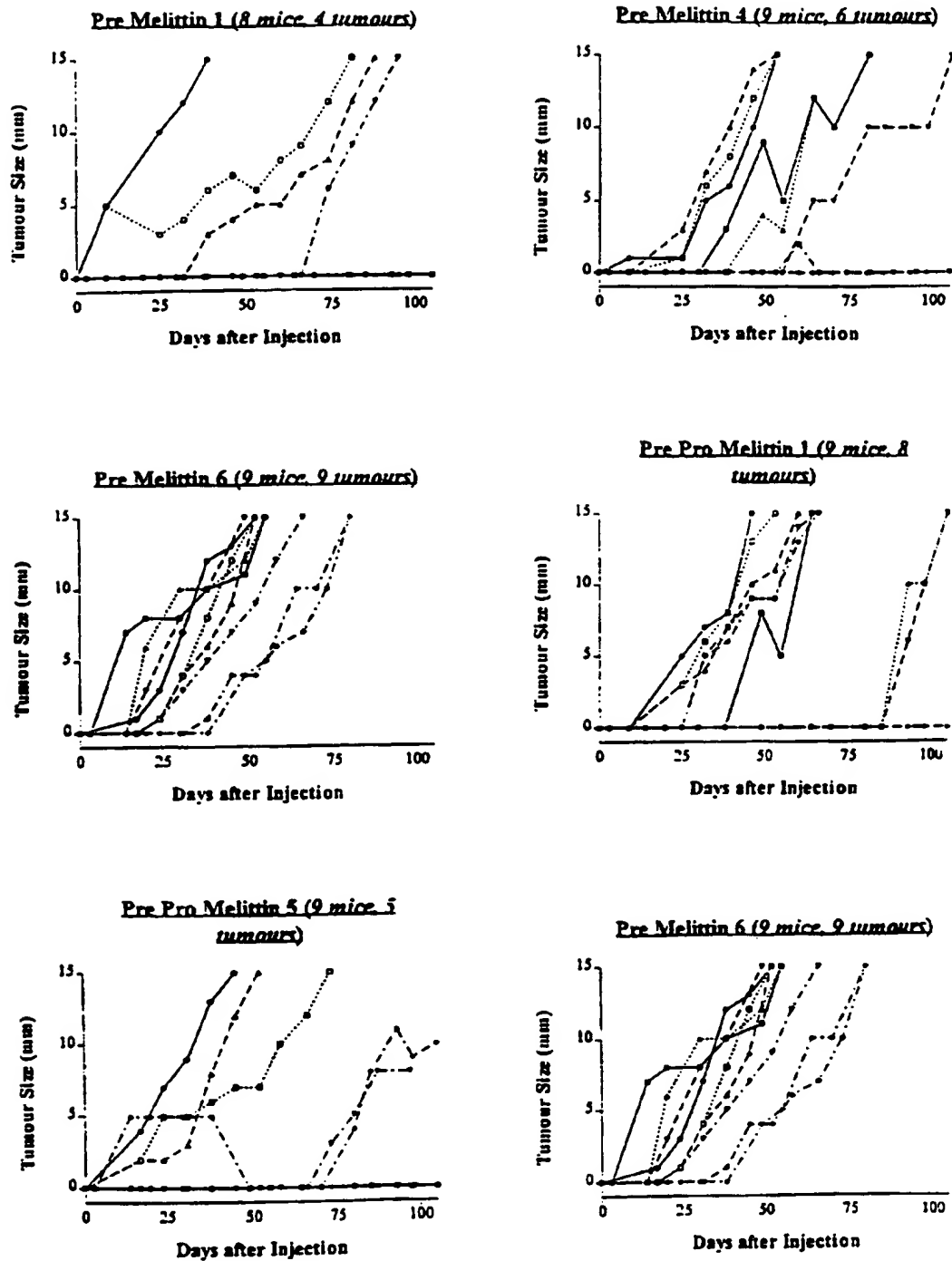


FIG 11

Effect of Peptides M & C on HIV Expression in EJ Cells

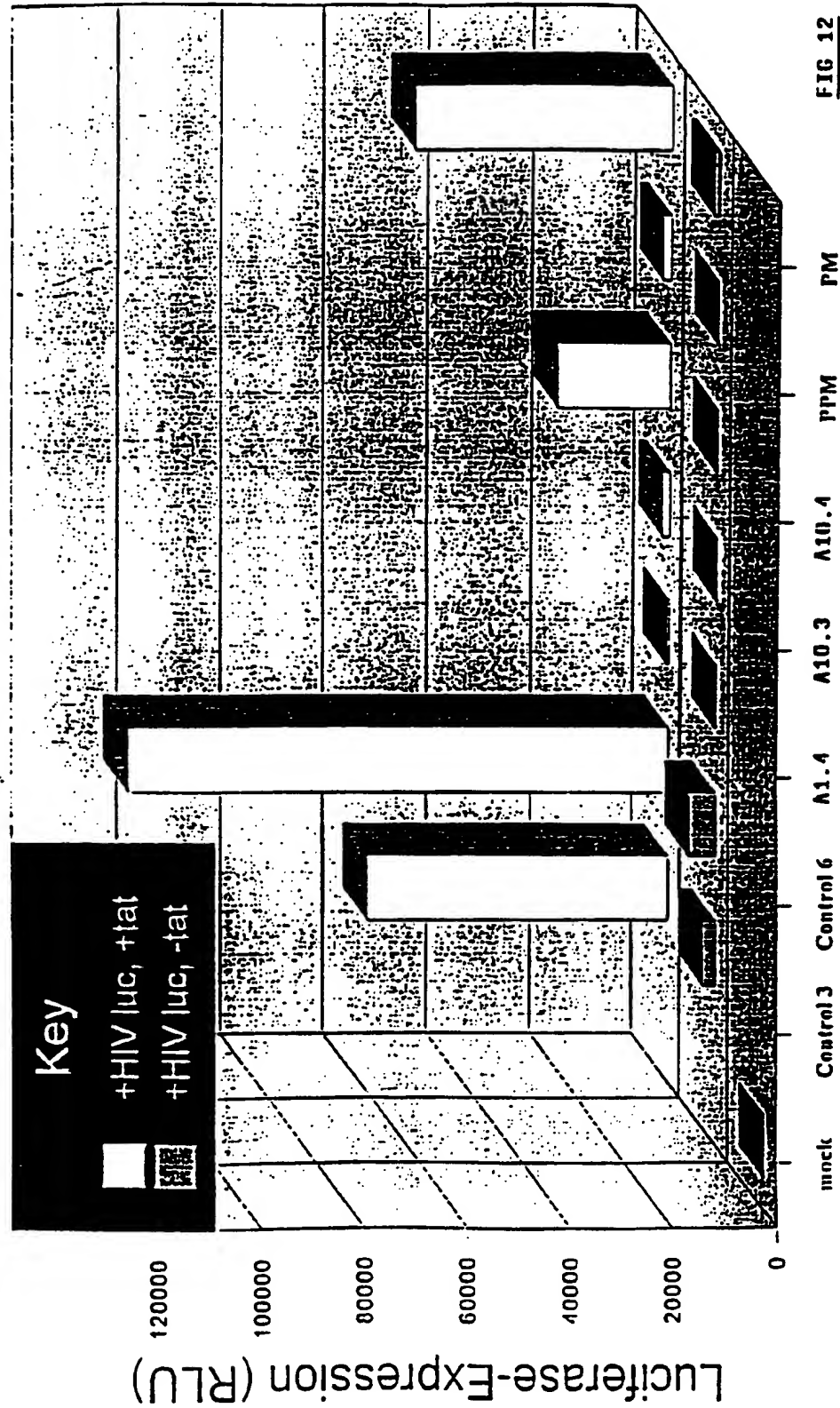


FIG 12

Percentage of HIV-driven control gene
expression (\pm tat) in cells supertransfected
with different clones expressing Cecropin

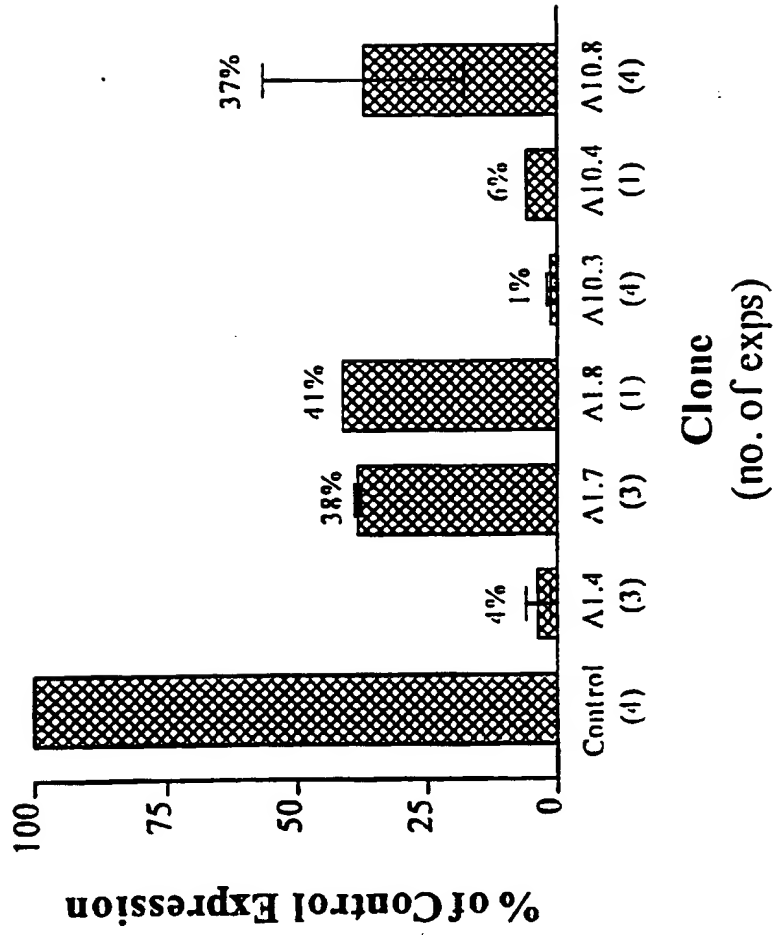
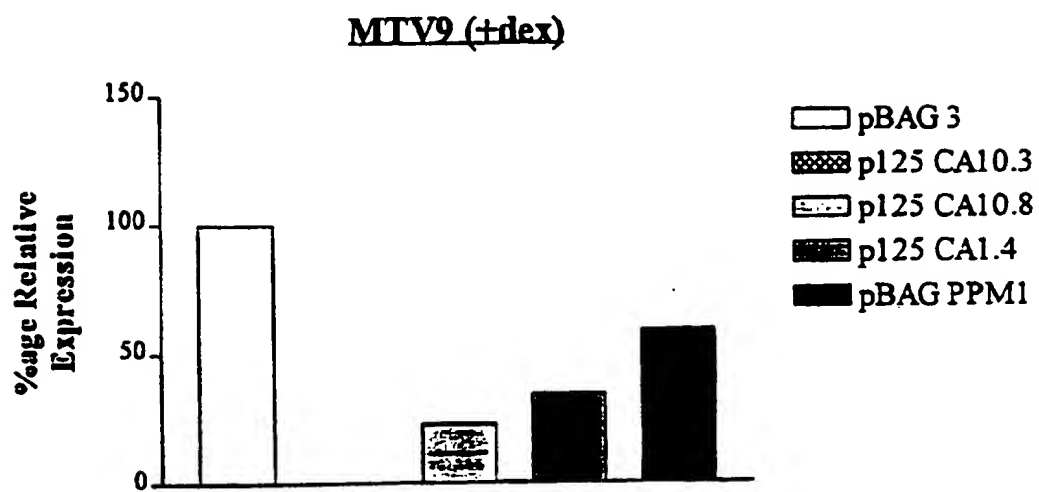
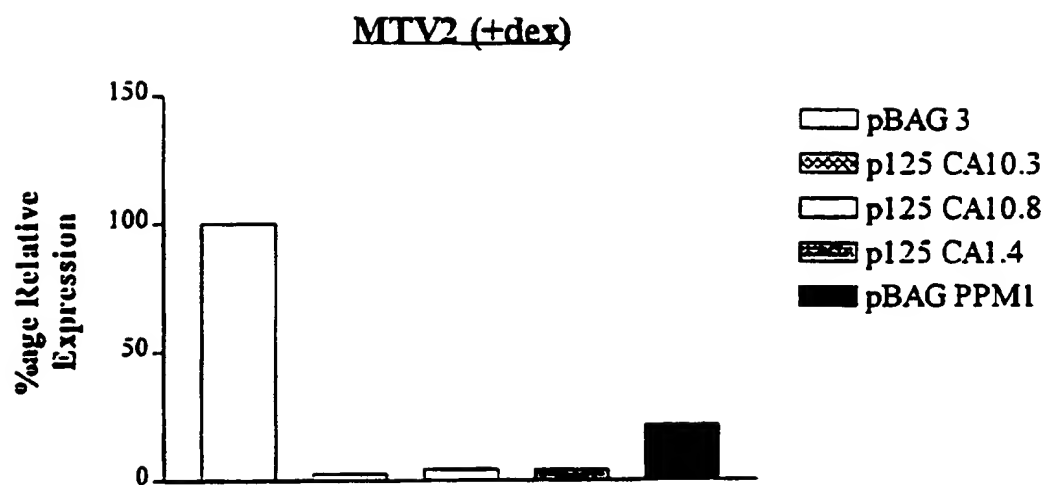


FIG 13

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**FIG 14**

INTERNATIONAL SEARCH REPORT

Int. Patent Application No

PCT/EP 96/01001

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/86 A61K38/17 A61K48/00 //C07K14/435

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | WO,A,93 07906 (SAN DIEGO REGIONAL CANCER CENTER) 29 April 1993 see page 73 - page 85 --- | 1,2 |
| Y | BIOLOGICALS, vol. 23, no. 1, March 1995, pages 5-12, XP002007572 W.H.GÜNZBURG ET AL.: "Retroviral vectors directed to predefined cell types for gene therapy" see the whole document --- | 1-10 |
| Y | WO,A,91 08753 (GESELLSCHAFT FÜR STRAHLEN- UND UMWELTFORSCHUNG MBH) 27 June 1991 see claims 9-13 --- -/- | 1-10 |

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- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

5 July 1996

Date of mailing of the international search report

25.07.96

Name and mailing address of the ISA

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Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/EP 96/01001

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | WO,A,95 01095 (BOARD OF SUPERVISORS OF LOUISIANA STATE UNIVERSITY AND AGRICULTURAL AN) 12 January 1995 see claims 46-48 --- | 40-42 |
| E | WO,A,96 07748 (GSF-FORSCHUNGSZENTRUM FÜR UMWELT UND GESUNHEIT GMBH) 14 March 1996 see the whole document ----- | 1-4, 13-23 |

1

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WEST

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 96/01001

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 29 - in so far it concerns an in vivo method - is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1-39 Retroviral vectors for gene therapy
 2. claims 40-42 Use of cecropins, maganinins or derivatives thereof in the manufacture of a medicament
-
1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
 2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
 3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
 4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 96/01001

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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